Reactive Oxygen Species Activation of Plant Ca^{2+} Channels. A Signaling Mechanism in Polar Growth, Hormone Transduction, Stress Signaling, and Hypothetically Mechanotransduction[1]

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Reactive oxygen species (ROS) are highly reactive reduced oxygen molecules. Recent studies have shown that production of ROS occurs in response to many physiological stimuli in plant cells, including pathogen attack, hormone signaling, polar growth, and gravitropism. Evidence is emerging that ROS can function as cellular second messengers that are likely to modulate many different proteins leading to a variety of responses. One target of ROS signal transduction is the activation of Ca^{2+}-permeable channels in plant membranes. ROS activation of Ca^{2+} channels may be a central step in many ROS-mediated processes, such as stress and hormone signaling, polar growth, development, and possibly during mechanotransduction.

MANY STIMULI INDUCE REACTIVE OXYGEN SPECIES IN PLANT CELLS

Apart from the well-recognized salicylic acid- and pathogen-induced ROS production (Chen et al., 1993; Lamb and Dixon, 1997; Torres et al., 2002), in recent years many additional stimuli have been shown to induce ROS production in plants. These include abscisic acid (ABA; Pei et al., 2000; Zhang et al., 2001), auxin (Joo et al., 2001; Schopfer et al., 2002), GAs (Fath et al., 2001), gravity (Joo et al., 2001), UV-B light (Mackerness et al., 2001), Nod factors (D'Haeze et al., 2003), and phytotoxins (Bais et al., 2003). How do ROS modify downstream targets? Many protein targets of ROS may exist, which could produce specific responses via ROS modification of proteins. ROS can modify protein structure and activity by causing the formation of disulfide bonds or sulfenic acid groups (Delaunay et al., 2002; Salmeen et al., 2003; van Montfort et al., 2003). We review here that one important ROS-signaling component is emerging in several plant signal transduction pathways, namely the activation of Ca^{2+}-permeable cation channels.

REACTIVE OXYGEN SPECIES, A PRIMER

ROS is the term used to describe the products of the sequential reduction of oxygen (O_2): one-electron reduction of O_2 forms the superoxide anion ($\cdot O_2^-$) and hydroperoxyl radical ($\cdot HO_2$; Fig. 1). A second one-electron reduction forms hydrogen peroxide (H_2O_2), and a third one-electron reduction produces the hydroxyl radical ($\cdot OH$; Fig. 1). Water is formed when $\cdot OH$ is further reduced (Fig. 1). The hydroperoxyl radical ($\cdot HO_2$) has a pKa value of 4.8 (Bielski et al., 1985), thus $\cdot HO_2$ and its deprotonated form, $\cdot O_2^-$, can both occur at slightly acidic pH, as found in cell walls. Unlike $\cdot HO_2$, H_2O_2 and $\cdot OH$ have high pKa values (11.6 and 11.9, respectively; Buxton et al., 1988); thus, the deprotonated forms of these compounds, HO_2^- and $\cdot O^-$, are usually negligible under physiological conditions.

Superoxide anion radicals ($\cdot O_2^-$) form H_2O_2 and O_2 spontaneously by a process termed dismutation or disproportionation. The rate of this reaction is rapid. The half-life of $\cdot O_2^-$ ranges from approximately 0.2 ms to 20 ms, assuming a concentration range of 10 μM to 1 mM $\cdot O_2^-$ (second order rate constant 5.4×10^6 M^{-1} s^{-1} at pH 6, calculated after Bielski et al., 1985). However, the enzyme superoxide dismutase further accelerates this reaction by approximately 400-fold (rate constant is 2.4×10^9 M^{-1} s^{-1}; Scandalios, 1997; Fig. 1). Thus, the typical life time of the superoxide anion is less than 1 ms.

H_2O_2 is a more stable ROS and can diffuse across membranes through water channels (Henzler and Steudle, 2000) and cause oxidative protein modifications at distal areas from its production (Scandalios et al., 1997). H_2O_2 forms $\cdot OH$ in the presence of transition metals such as iron and copper (Fenton reaction; Halliwell and Gutteridge, 1999; Fig. 1).

The reactivity of the hydroxyl radical ($\cdot OH$) is very high (rate constants for many biological molecules are 10^8–10^{10} M^{-1} s^{-1}; Buxton et al., 1988). The half-life of $\cdot OH$ may therefore be in the nanosecond range in

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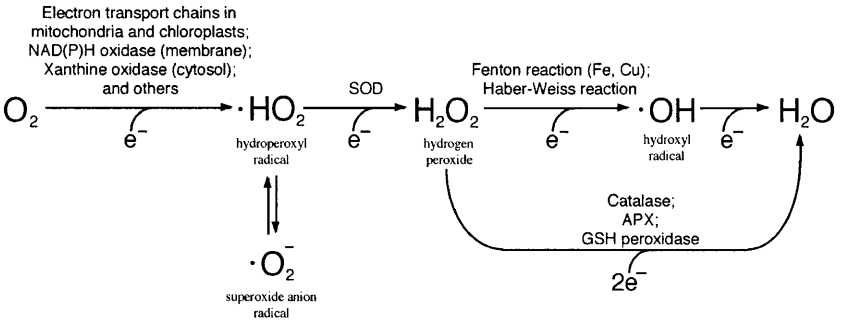

Figure 1. Metabolic pathways of reactive oxygen species in plants. Some of the important enzymes in reactive oxygen species metabolic pathways are illustrated. APX, ascorbate peroxidase; GSH, glutathione; SOD, superoxide dismutase.

cells. Therefore, it is not possible for ·OH to migrate in solution; instead, ·OH will react with itself, other ROS, or with proteins, lipids, and other biomolecules in close proximity to ·OH production. Thus, ·OH can play a role as a localized reaction intermediate, but it generally cannot transduce a signal to a more distant target molecule.

ROS REGULATION OF HYPERPOLARIZATION-DEPENDENT PLASMA MEMBRANE Ca^{2+}-PERMEABLE CATION CHANNELS

ROS induce cytosolic Ca^{2+} increases in guard cells and stomatal closure in Commelina and Arabidopsis (McAinsh et al., 1996; Pei et al., 2000). ROS activation of a hyperpolarization-dependent Ca^{2+}-permeable cation (I_{Ca}) channel was identified in the plasma membrane of Arabidopsis guard cells (Pei et al., 2000). ROS levels in guard cells increase in response to ABA application (Pei et al., 2000; Zhang et al., 2001). ROS activation of I_{Ca} channels is impaired in the recessive ABA insensitive *gca2* mutant (Pei et al., 2000) and also in the dominant ABA insensitive *abi2-1* protein phosphatase mutant (Allen et al., 1999; Murata et al., 2001), thus providing molecular genetic evidence for the relevance of I_{Ca} channel activation in ABA signal transduction.

Biochemical studies showed that recombinant ABI1 and ABI2 protein phosphatase 2C (PP2C) activities are inhibited by H_2O_2, which indicates that these PP2Cs may represent direct targets of ROS in ABA signaling (Meinhard and Grill, 2001; Meinhard et al., 2002). ROS inhibition of the ABI1 and ABI2 PP2Cs is consistent with the model that ABI1 and ABI2 function as negative regulators of ABA signal transduction (Sheen, 1998; Merlot et al., 2001). ABA inhibition of negatively regulating PP2Cs could contribute to turning up the ABA signaling pathway. Whether ROS also directly modify the I_{Ca} channel proteins and/or additional intermediate regulatory proteins remains to be determined.

ROS ACTIVATION OF CALCIUM CHANNELS: A BROADLY USED SIGNALING CASSETTE

Recent reports have identified and characterized a class of hyperpolarization-activated Ca^{2+}-permeable cation channels in several types of plant cells, including tomato (*Lycopersicon esculentum*) suspension culture cells (Gelli and Blumwald, 1997), guard cells (Hamilton et al., 2000; Pei et al., 2000), root hair cells (Véry and Davies, 2000), root elongation zone epidermal cells (Kiegle et al., 2000; Demidchik et al., 2002a, 2003; Foreman et al., 2003), and algal rhizoid cells (Coelho et al., 2002). In tomato suspension culture cells, fungal elicitor activation of I_{Ca}-type Ca^{2+} channels (Gelli et al., 1997) is inhibited by the antioxidant, dithiothreitol (A. Gelli and E. Blumwald, personal communication), indicating a possible role for ROS in channel activation.

Elicitors evoke both cytosolic Ca^{2+} increases and ROS generation; however, the peptide elicitor harpin induces only ROS generation (Chandra and Low, 1997). In some cases, Ca^{2+} elevations have been reported upstream of ROS production; in other cases, Ca^{2+} elevations occur downstream of ROS production (Bowler and Fluhr, 2000), indicating complex spatiotemporal Ca^{2+} elevation mechanisms. In tobacco (*Nicotiana plumbagifolia*) seedlings, oxidative stress stimulates cytosolic Ca^{2+} increases (Price et al., 1994). A recent study showed that the allelopathic toxin (−)-catechin causes rapid ROS production, followed by ROS-induced Ca^{2+} increases in *Centaurea diffusa* and Arabidopsis roots, suggesting a broader role for ROS-Ca^{2+} signaling in pathogenic responses (Bais et al., 2003).

But which enzymes of the many ROS producing and scavenging proteins (Mittler, 2002) cause signal-induced ROS production? In mammalian systems, growth factors such as epidermal growth factor and platelet-derived growth factor stimulate ROS generation (Sundaresan et al., 1995; Bae et al., 1997). Reactive oxygen species reversibly inhibit protein Tyr phosphatase activity by oxidizing a Cys residue in the catalytic site (Lee et al., 1998; Rhee et al., 2000; Salmeen et al., 2003; van Montfort et al., 2003). ROS inhibition of these negatively regulating phosphatases enhances stimulation of Tyr phosphorylation by the epidermal growth factor and platelet-derived growth factor receptor Tyr kinases (Salmeen et al., 2003; van Montfort et al., 2003). However, in mammalian cells the ROS-producing enzymes that mediate growth factor-induced ROS production remain unknown. In plant cells, 10 different possible mechanisms of ROS production are known (Mittler, 2002), including mitochon-

drial and chloroplast electron transfer, membrane bound NAD(P)H oxidases, and cytosolic xanthine oxidase (Fig. 1).

NAD(P)H OXIDASES AS MEDIATORS OF ROS-I_{Ca} SIGNALING IN ROOT HAIR POLAR GROWTH AND GUARD CELLS

In Fucus rhizoid cells, there is a local oxidative burst at the growing rhizoid tip (Coelho et al., 2002). Furthermore, ROS activation of rhizoid apex Ca^{2+} channels and a tip-focused Ca^{2+} gradient after hyperosmotic treatment were demonstrated (Coelho et al., 2002). The pharmacological NAD(P)H oxidase inhibitor, diphenylene iodinium (DPI), inhibited tip growth in Fucus and the tip-localized Ca^{2+} gradient. DPI also partially inhibits ABA-induced stomatal closing (Pei et al., 2000).

Recently, direct genetic evidence was obtained for a function of membrane bound NAD(P)H oxidases in root hair growth and ABA-ROS signal transduction in guard cells. Hyperpolarization-activated Ca^{2+} channels are activated by the hydroxyl radical (·OH) in epidermal cells of the Arabidopsis root elongation zone (Foreman et al., 2003). Loss-of-function mutations in the NAD(P)H oxidase gene, *atrbohC* (also named *rhd2*, for *root hair defective2*), caused a short root hair phenotype (Foreman et al., 2003). Polar growth is associated with tip-localized Ca^{2+} influx and cytosolic Ca^{2+} elevations (Malho and Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli et al., 2000; Plieth and Trewavas, 2002). Interestingly, the root hair tip-focused Ca^{2+} gradient and root hair bulge-localized ROS elevations were impaired in *atrbohC*. Exogenous application of ·OH to roots in the *atrbohC* mutant induced spherical (nonpolar) root hair bulges (Foreman et al., 2003). In contrast in a different study, Arabidopsis root hair growth rate was attenuated with the application of H_2O_2, which induced $[Ca^{2+}]_{cyt}$ elevation (Jones et al., 1998). This apparent difference in ROS responses may be explained by the different developmental stages of root hairs and/or the lack of tip-focused ROS production when exogenous ROS are applied. The characterization of the *atrbohC* mutant demonstrates a role for ROS in mediating root hair growth.

Direct evidence was lacking that ROS function as rate-limiting second messengers during guard cell ABA signal transduction. Two catalytic subunit genes encoding NAD(P)H oxidases, *AtrbohD* and *AtrbohF*, were found to be highly expressed in guard cells, and both mRNAs are elevated in response to ABA (Kwak et al., 2003). Double knockout of the partially redundant NAD(P)H oxidases showed ABA insensitivity of stomatal closing and impairment in both ABA induction of ROS accumulation and ABA activation of I_{Ca} channels (Kwak et al., 2003). NAD(P)H oxidases produce $\cdot O_2^-$, which readily forms H_2O_2 (Fig. 1). Exogenous H_2O_2 application restored I_{Ca} channel activation and partial stomatal closing in the *atrbohD atrbohF* double mutant. These findings identify NAD(P)H oxidases as important mediators of ABA-induced ROS production and ABA activation of I_{Ca} channels. Consistent with this hypothesis, in guard cells, elicitors that cause ROS production and stomatal closing (Lee et al., 1999) activate I_{Ca} channels in a cytosolic NAD(P)H-dependent manner (Klüsener et al., 2002).

Importantly, the linkages of NAD(P)H oxidases to ROS production and ROS activation of Ca^{2+} channels in Arabidopsis roots (Foreman et al., 2003), in Fucus rhizoids (Coelho et al., 2002), and in guard cells (Pei et al., 2000; Kwak et al., 2003) indicate that the ROS-I_{Ca} channel pathway may represent a more widely used signaling cassette (McAinsh and Hetherington, 1998) in plant biology.

In addition to NAD(P)H oxidases, other classes of ROS producing and scavenging enzymes (Mittler, 2002) are likely to contribute to ROS regulation of I_{Ca} channels during signal transduction and development. Furthermore, ion channels often function as signaling nodes upon which parallel signal transduction pathways converge (Hille, 1992; Assman and Shimazaki, 1999; Schroeder et al., 2001; Sanders et al., 2002). Therefore, it is likely that I_{Ca} channels are regulated by additional parallel mechanisms. In Arabidopsis mesophyll cells, blue light activates an I_{Ca}-like Ca^{2+} current (Stoelzle et al., 2003). Blue light activation of I_{Ca}-like Ca^{2+} channels was proposed not to require ROS production based on lack of an inhibitory effect of the pharmacological NAD(P)H oxidase inhibitor DPI (Stoelzle et al., 2003). Moreover, a second type of I_{Ca}-like Ca^{2+} current exists in root hairs, which was reported not to be ROS regulated (Véry and Davies, 2000; Demidchik et al., 2003). Protein kinase and phosphatase inhibitors have been shown to modulate I_{Ca} channels in *Vicia faba* guard cells (Köhler and Blatt, 2002). Future research will show whether phosphorylation events and other possible I_{Ca} channel regulators function parallel to ROS production or sequentially in the same signaling branch.

CAN MECHANOSENSING CHANNELS BE STIMULATED VIA ROS PRODUCTION?

Mechanosensing in plants remains an elusive field. Stretch-activated channels have been proposed to function as general mechanosensors in signal transduction (Falke et al., 1988). However, relatively few studies of stretch-activated channels in plants have been reported (e.g. Falke et al., 1988; Cosgrove and Hedrich, 1991; Ding and Pickard, 1993a, 1993b), and the molecular mechanisms underlying stretch activation of channels in plants remain largely unknown. While several channel types in plants are indeed modulated by membrane stretch, which can contribute to mechanosensing, no genetic evidence for their functions in mechanosensing has yet been obtained.

As reviewed above, recent studies in Fucus rhizoids and Arabidopsis root hairs revealed that polar growth is associated with tip-localized ROS elevation and is correlated with ROS activation of Ca^{2+}-permeable channels (Coelho et al., 2002; Foreman et al., 2003). Hyperosmotic stress of Fucus rhizoids also leads to ROS production and tip-focused cytosolic Ca^{2+} elevation (Coelho et al., 2002).

The tip-focused Ca^{2+} influx during polar growth has long been hypothesized to be mediated by stretch-activated nonselective cation channels (for reviews and references, see Boonsirichai et al., 2002; Demidchik et al., 2002b; Robinson and Messerli, 2002; Perbal and Driss-Ecole, 2003). Alternatively, polar tip growth and tropic responses may be mediated by developmental pathways and/or local mechanical stresses, which in turn cause ROS production and oxidative bursts. Such oxidative bursts could then activate plasma membrane I_{Ca} channels. This working hypothesis would suggest focusing early mechanosensing analyses on mechanisms that modulate ROS-producing enzymes, in order to elucidate upstream mechanosensors and ROS producer activation mechanisms. Note that this hypothesis does not necessarily exclude possible parallel direct mechanical activation of stretch-activated channels.

Interestingly, recent research in vascular smooth muscle has suggested that mechanical stretch induces ROS production by activation of NAD(P)H oxidases (Grote et al., 2003). But how could mechanostimulation be translated into regulation of ROS generating enzymes? Conceivably, mechanostimulation may modulate ROS producing enzymes via interaction with the cytoskeletal network and/or cell walls. Stretch-activated channels have been hypothesized to be activated by tension via actin filaments that are deformed by statoliths during root gravisensing (Perbal and Driss-Ecole, 2003). Polar growth of Arabidopsis root hairs is perturbed by actin-depolymerizing and microtubule depolymerizing drugs (for review, see Hepler et al., 2001; Ketelaar et al., 2003). Mutations in the actin-related proteins 2 and 3, which are the major subunits of the Arp2/3 complex, result in cell shape defects, for example during leaf epidermal cell development, root hair growth, and trichome development (Frank and Smith, 2002; Li et al., 2003; Mathur et al., 2003; Van Gestel et al., 2003). However, whether ROS producing enzyme activities are modulated by cytoskeletal networks remains to be examined during polar growth or mechanical stimulation.

LOCALIZED REGULATION OF ROS PRODUCTION AND NAD(P)H OXIDASES

As discussed above, recent genetic studies have linked NAD(P)H oxidase genes to polar growth and ABA-I_{Ca} channel signaling (Foreman et al., 2003; Kwak et al., 2003). In mammalian cells, NAD(P)H oxidases are composed of the plasma membrane catalytic subunits $gp91^{phox}$ (homologs of *Atrboh*) and $p22^{phox}$ proteins, which form a heterodimeric flavocytochrome (Diebold and Bokoch, 2001). During activation in phagocytes, two cytosolic proteins, p47 and p67, and the small G protein Rac translocate to the plasma membrane, resulting in formation of the active NAD(P)H oxidase complex (Diekmann et al., 1994; Diebold and Bokoch, 2001). Interestingly, no homologs of the mammalian p47 and p67 NADPH oxidase subunits are found in the Arabidopsis genome (Torres et al., 2002), suggesting that elucidation of unique regulation mechanisms of plant NAD(P)H oxidases is needed.

In guard cells, the ABA-insensitive *abi1-1* PP2C and *ost1* protein kinase mutants and phosphatidylinositol3-kinase inhibitors all impair ABA-induced ROS production, indicating that these protein phosphorylation-related enzymes and phosphatidylinositol3-phosphate may directly or indirectly regulate ROS production proteins (Murata et al., 2001; Mustilli et al., 2002; Park et al., 2003).

A previous study suggested that activation of the small G proteins, AtROP2 and AtROP1 (also named AtRac11), is required for polar growth of Arabidopsis root hairs (Jones et al., 2002) and pollen tubes (Li et al., 1999). Furthermore, AtROP1, AtROP2, and NtROP1 regulate actin bundle formation in growing tips of Arabidopsis and tobacco (Fu et al., 2001, Jones et al., 2002; Chen et al., 2003). Raising the extracellular Ca^{2+} concentration rescues pollen tube growth inhibition in antisense *atrop1* and dominant negative *atrop1* plants (Li et al., 1999). These results support the hypothesis of a relationship between AtROP/Rac small G proteins, the cytoskeletal network, and regulation of tip-localized Ca^{2+} influx in polar growth.

Some plant NAD(P)H oxidases are targeted to the plasma membrane (Keller et al., 1998; Sagi and Fluhr, 2001). Therefore, it is conceivable that signal transduction mechanisms may activate NAD(P)H oxidases causing local ROS production and subsequent local activation of Ca^{2+}-permeable channels in plant membranes. Note that in *V. faba* guard cells, exogenous H_2O_2 inhibits outward K^+ channels (Köhler et al., 2003), which would inhibit ABA-induced stomatal closing. This finding and the function of NAD(P)H oxidases in ABA-induced stomatal closing (Kwak et al., 2003) together indicate that a localized oxidative burst may occur in response to ABA, similar to observations at the Fucus rhizoid tip (Coelho et al., 2002). Further research is needed to determine by which mechanisms NAD(P)H oxidases and ion channels are biochemically linked to ensure specificity in ROS regulation and localized ROS production (see Kwak et al., 2003).

CONCLUSIONS

Recent findings have shown that many stimuli cause ROS production in plant cells and that ROS activate plasma membrane I_{Ca} channels and cytosolic

Ca^{2+} elevations in several plant cell types. Moreover, membrane-bound NAD(P)H oxidases function in root hair growth and in guard cell ABA activation of I_{Ca} channels, providing direct genetic evidence that ROS generation is rate-limiting for Ca^{2+} signaling during these responses. We extrapolate from these findings and propose a testable working hypothesis that ROS production may also contribute to mechanotransduction in plants. Further analyses of the ROS-I_{Ca} channel signaling cassette may bring new surprises and shed light into long-standing questions in plant physiology.

Note Added in Proof

Two recent publications provide further data showing links between ROS production and stomatal closing. Chen and Gallie (2004) showed a relationship between ascorbic acid levels, guard cell redox state, and stomatal aperture. Suhita et al. (2004) reported that methyl jasmonate treatment caused ROS production in guard cells and stomatal closing. These responses were impaired in the *atrbohD/atrbohF* and the methyl jasmonate-insensitive *jar1* mutants (Suhita et al., 2004).

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